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Research Article

# Molecular phylogenetic analysis of *Neritona juttingae* (Mienis, 1973) (Gastropoda, Cycloneritida, Neritidae) with remarks on the phylogenetic position of the genus *Neritona*

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## Abstract

In this study, the phylogenetics of an ornamental neritid snail, *Neritona juttingae* (Mienis, 1973), a species native to Southeast Asia and commercially distributed in Chinese markets are studied for the first time. The first complete mitochondrial genome and nuclear ribosomal RNA gene cluster are reported. Phylogenetic trees based on all mitochondrial genes strongly support the division of Neritidae into two subfamilies: Neritinae and Neritininae. Our phylogenetic analysis placed *N. juttingae* within Neritininae, where it forms a distinct evolutionary lineage. The monophyly of the genus *Neritona* remains unresolved because only one species is involved. The relationships among genera within Neritininae were poorly resolved because of the discordant topologies across datasets. Our investigation of mitochondrial gene order in Neritidae revealed highly conserved arrangements. How taxonomic misidentifications and labeling errors in public databases may impact phylogenetic inferences is discussed. This research provides baseline data for aquatic ornamental pet species, contributes to a better understanding of the phylogenetic relationships within Neritoidea and highlights some problems in phylogenetic analysis.

**Key words:** 16S rRNA, *COI*, genome skimming, mitogenome, *Neritona juttingae*, phylogenetic analysis, ribosomal RNA gene cluster

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## Introduction

*Neritona juttingae* (Mienis, 1973) is a freshwater gastropod species belonging to the family Neritidae (Cycloneritida: Neritoidea), distributed in Southeast Asia (Mienis 1973; Eichhorst 2016a). It has an atypical appearance, with 6–9 spiral cords on the semi-globose shell, and the cords have a series of small nodules or spines, which makes it very popular among shell collectors and aquarium hobbyists (Eichhorst 2016a, b; Ng et al. 2016). As a result, the species has entered commercial trade and is currently available in ornamental pet markets around the world (Ng et al. 2016; Schäfer 2018; this study).

Despite being popular among hobbyists, hardly any studies on this species exist. The original name of this species is *Nerita aculeata* Gmelin, 1791. Subsequently, Sowerby II (1849) placed it in the genus *Neritina* Lamarck, 1816. Mienis (1973) proposed that the name of this species was preoccupied: *Nerita aculeata* Gmelin, 1791 is a junior homonym of *Nerita aculeata* Muller, 1774 [a junior synonym of *Tympanotonos fuscatus* (Linnaeus, 1758)], and renamed it as *Neritina juttingae* Mienis, 1973 (Eichhorst 2016a). However, *Neritina* was a "dustbin" genus that contained most freshwater or brackish neritid species in the past. Some researchers have proposed different classifications to split *Neritina* into multiple genera or subgenera, but these have not been widely accepted. Eichhorst (2016a, b) proposed a comprehensive classification based on shell morphology and the previous studies after revising all extant Neritidae species. He formally classified Neritidae as two subfamilies with 16 genera: Neritinae Rafinesque, 1815, and Theodoxinae Bandel, 2001 (= Neritininae Poey, 1852). Neritinae contains two marine genera, *Nerita* Linnaeus, 1758, and *Mienerita* Dekker, 2000; Neritininae contains 14 genera, most of which are freshwater or brackish, *Neritona* von Martens, 1869 being one of them.

Currently, the genus *Neritona* comprises only six valid species: *Neritona granosa* (G. B. Sowerby I, 1825); *N. juttingae* (Mienis, 1973); *N. labiosa* (G. B. Sowerby I, 1836) (type species); *N. macgillivrayi* (Reeve, 1855); *N. melanesica* Riech, 1935; *N. plannissima* (Musson, 1869). Von Martens originally established the genus *Neritona* based on the morphology of the operculum of *N. labiosa* (von Martens, 1869). Eichhorst (2016a) redefined the genus, based on the following characters: 1) developed corneous lamella on the operculum; 2) long, thin, and curved apophysis, and long and slender peg; 3) large and semi-globose shell. Most members of this genus are distributed from Southeast Asia to S. Pacific regions, but *N. granosa* is endemic to Hawaii (Eichhorst 2016a).

To date, the monophyletic status and phylogenetic position of this genus remain unexplored based on molecular data. Although several phylogenetic studies based on mitochondrial genomes have been conducted in recent years (Frey and Vermeij 2008; Quintero-Galvis and Raquel-Castro 2013; Sands et al. 2020; Feng et al. 2021; Miao et al. 2022), all of these studies lack sequence data for the genus *Neritona*. Currently, only two studies have provided sequence data for the genus: Alda et al. (2016) sequenced 606 individuals of *N. granosa* to investigate their *COI* haplotypes; Ng et al. (2016) provided the partial mitochondrial cytochrome oxidase subunit I (*COI*) and 16S ribosomal RNA (16S) sequences of *N. juttingae* in an assessment of ornamental freshwater mollusks in Singapore.

In this study, we generated molecular data for *Neritona juttingae* using the genome skimming methods. The molecular data included the complete mitochondrial genome and complete nuclear ribosomal DNA sequences (18S-ITS1-5.8S-ITS2-28S). The phylogenetic position of *Neritona juttingae* was analyzed by combining the new sequence data with all available mitochondrial genomes and partial mitochondrial *COI* and 16S sequences of Neritidae from NCBI, and the monophyly of *Neritona* was also discussed.

## Materials and methods

### Specimen collection, identification, and sequencing

The sequenced specimen was bought from Chinese aquarium trading establishement, collected from Sumatra, Indonesia (Fig. 1). Morphological identification followed Eichhorst (2016a). To further confirm the taxonomic identity, we conducted online BLAST analyses (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome) using default parameters, based on the mitochondrial genome assembled from genome skimming data. In addition, we reconstructed phylogenetic trees incorporating all available *COI* and 16S sequences of Neritidae from NCBI. A detailed method of the BLAST analysis is provided in Yang et al. (2024). The specimen was fixed in ethanol (≥ 99.7%), preserved in a -20 °C refrigerator, and finally deposited at the College of the Environment and Ecology, Xiamen University (number: RTX-NER-01).

Specimens were washed with ≥ 97% ethanol before DNA extraction to minimize potential contamination. To avoid intestinal contamination, a tissue sample was clipped from the foot of the specimen. Whole genomic DNA was extracted using the TIANamp Genomic DNA Kit (TIANGEN, Beijing, China). Genomic sequencing was conducted by Novogene Bioinformatics Technology Co., Ltd. (Beijing, China), using a paired-end 150 bp (PE150) strategy on the Illumina NovaSeq X Plus platform. This process yielded 5.60 Gb of raw sequencing data. The raw data were deposited in NCBI under the BioProject accession number PRJNA1394617: BioSample accession SAMN54324965, Sequence Read Archive (SRA) accession SRR36610501.

### Assembly and annotation of mitochondrial genome

The raw paired-end sequencing reads were processed with fastp v. 0.23.4 (Chen et al. 2018) to remove sequencing adapters and trim low-quality regions. To ensure reliable mitochondrial genome assembly, we followed our established assembly pipelines by Yang et al. (2024). In this study, we employed three different assembly tools: (1) NovoPlasty v. 4.3.1 (Dierckxsens et al. 2017), using all mitochondrial genome sequences of Neritidae as seed (NCBI database, September 10, 2024) with default parameters; (2) GetOrganelle v. 1.7.6.1 (Jin et al. 2020), with k-mer thresholds set to 17, 21, 33, 39, 45, 55, 65, 75, 85, 95, 105, 115, and 127, while other parameters were set to default; and (3) FastMitoAssembler (available at https://github.com/suqingdong/FastMitoAssembler). All three methods produced circular mitochondrial genome sequences, differing only in the control region. FastMitoAssembler integrates the approaches of MEANGS, NovoPlasty, and GetOrganelle (Yang et al. 2024), its assembly was ultimately selected, resulting in a mitochondrial genome of 15,950 bp.

The assembled mitochondrial genome was annotated using three tools: MITOS2 (Donath et al. 2019), MitoFinder v. 1.4.1 (Allio et al. 2020), and MitoZ v. 3.6. The generated annotation files (.gb or .gbf) were reorganized with *cox1* (*COI*, *COX1*) as the starting gene using PhyloSuite v.1.2.3 (Zhang et al. 2020). The restructured GenBank (.gb) files were then converted into FASTA format and subjected to tRNA annotation using GeSeq (https://chlorobox.mpimp-golm.mpg.

de/geseq.html). Annotations were manually checked using Geneious Prime v. 2022.2.2 (Kearse et al. 2012), following the annotation principles outlined by Yang et al. (2024). The finalized annotation was further confirmed by aligning with mitochondrial genome sequences of closely related species.

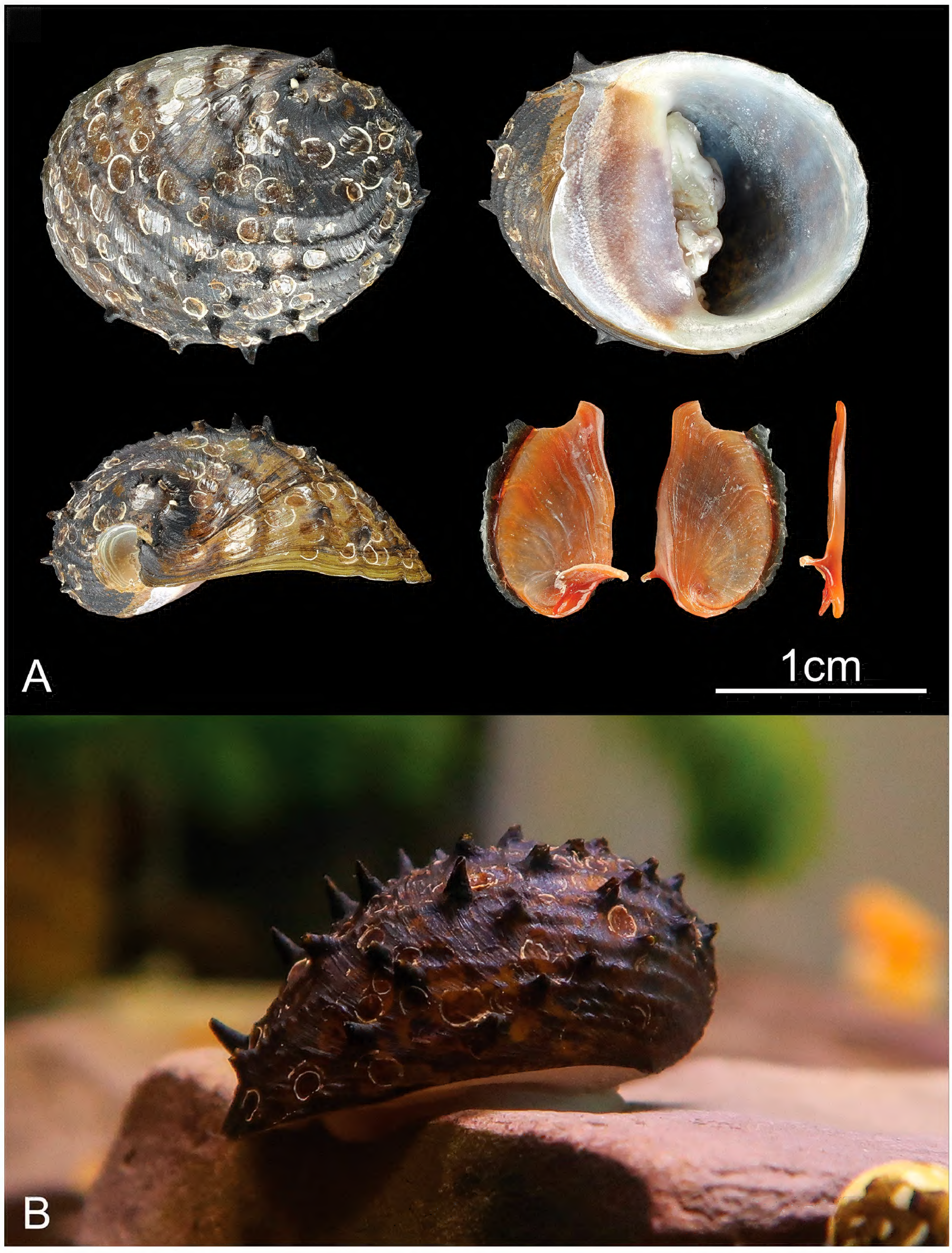


**Figure 1.** Specimens of *Neritona juttingae*. **A.** RTX-NER-01, shell and operculum; **B.** A living individual of *N. juttingae*. Photographed by Y. Z. M.

The annotated mitochondrial genome sequence was submitted to the NCBI GenBank database with accession number PP958837. The GC content and nucleotide coverage depth across the mitochondrial genome were calculated using the 'visualize' subcommand in MitoZ v. 3.6 (Meng et al. 2019), and the gene map illustrating the annotated mitochondrial genome was also generated (Fig. 2).

## Assembly of nuclear ribosomal RNA gene cluster

The complete nuclear ribosomal RNA gene cluster (18S-28S rRNA) was assembled using GetOrganelle v. 1.7.6.1 (Jin et al. 2020), with sequence seeding performed using all available 18S and 28S rRNA sequences from Gastropoda downloaded from GenBank as of 15 June 2025. The assembled sequence spans 10,124 bp (accession number: PV833224) with an average coverage depth of 430.9×. Sequence annotation was performed using Geneious Prime v. 9.0.2 with the “Live Annotate & Predict” function enabled. The internal transcribed spacer regions (ITS1 and ITS2) were identified based on the boundaries flanking the 18S, 5.8S, and 28S ribosomal RNA genes.

## Phylogenetic analysis

The newly sequenced mitochondrial genome of *Neritona juttingae* (PP958837) was analyzed alongside all available sequences of Neritidae (36 sequences from the NCBI database, October 1, 2024) using the phylogenetic methods described below, based on a dataset of 13 protein-coding genes (13PCGs). Our analysis revealed that sequences belonging to the same species consistently formed monophyletic groups (Suppl. material 2). We selected a single representative sequence for each species to simplify the analysis. The final

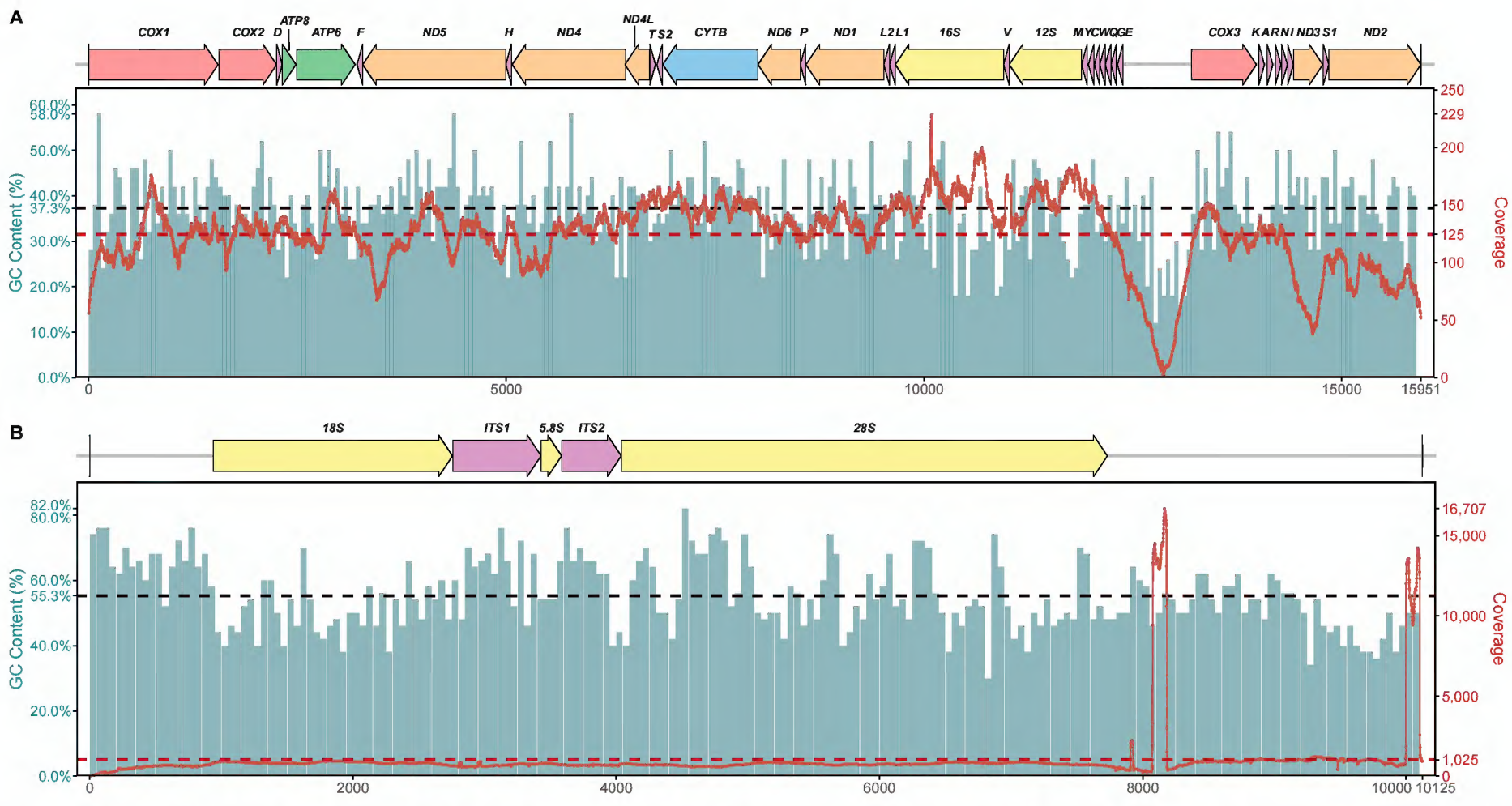


**Figure 2.** The molecular resources of *Neritona juttingae*. The gene map of mitochondrial genome (**A**) and nuclear rRNA cluster (**B**). The red lines depict the distribution of coverage depth and the green columns depict the GC-content of sequences.

dataset, summarized in Table 1, comprised 29 species of Neritidae and two outgroup taxa of Cycloneritida, *Titiscania limacina* (KU342669) and *Georissa bangueyensis* (KU342664), belonging to Neritopsidae and Hydrocenidae (Qi et al. 2023).

The extracted protein-coding genes (PCGs) and two rRNAs (2R) from these sequences were aligned using MAFFT in normal mode. Improvement of the multiple sequence alignments for the 13 PCGs was performed using MACSE v. 2 (Ranwez et al. 2018) under the "refinement strategy." Phylogenetic analyses were conducted on five datasets: (i) 13PCGs123, including all three codon positions of the 13 PCGs; (ii) 13PCGs123+2R, combining the 13PCGs dataset with two rRNA genes; (iii) 13PCGs12, excluding the third codon positions of the 13 PCGs; (iv) 13PCGs12+2R, combining the 13PCGs12 dataset with two rRNA genes; and (v) 13PCGsAA, comprising amino acid sequences translated from the 13 PCGs. To include a broader representation of Neritidae species, sequences of the widely used molecular markers *COI* and 16S rRNA were extracted from GenBank. Data organization was performed using a script developed by Yang et al. (2024). The final combined *COI* and 16S dataset comprised 114 species representing 12 genera of Neritidae, along with two outgroup taxa, totaling 257 *COI* and 257 16S sequences (Suppl. material 1: table S1). Additionally, more *COI* sequences were used for phylogenetic analysis (*COI*_ex dataset).

TrimAl v. 1.2 (Capella-Gutiérrez et al. 2009) was used to remove ambiguously aligned regions with the 'automated1' setting (Suppl. material 1: table S2). Substitution models were selected using ModelFinder v. 2.2.0 (Kalyaanamoorthy et al. 2017) based on the Bayesian Information Criterion (BIC) for maximum likelihood (ML) analysis and the corrected Akaike Information Criterion (AICc) for Bayesian inference (BI) analysis under a partitioned model (Suppl. material 1: table S3). ML phylogenetic reconstruction was performed using IQ-TREE v. 2.2.2 (Nguyen et al. 2015; Kalyaanamoorthy et al. 2017) with the best partition scheme and an edge-linked partition model. Node support was evaluated with 200,000 ultrafast bootstraps. BI phylogenies were constructed in MrBayes v. 3.2.7a (Ronquist et al. 2012), with two parallel runs and 2,000,000 generations. Convergence was assessed by the average standard deviation of split frequencies (ASDSF), with additional generations added if the ASDSF value exceeded 0.01.

Topological differences among the inferred phylogenies were evaluated using TreeSpace (Jombart et al. 2017) implemented in R v. 4.3.1 (R Core Team 2023). Finally, the ML and BI trees were visualized using iTOL v. 6 (Letunic and Bork 2024).

## Sequence analyses

Strand asymmetries were calculated using the formulas proposed by Perna and Kocher (1995): AT-skew = (A - T) / (A + T) and GC-skew = (G - C) / (G + C). Codon usage and relative synonymous codon usage (RSCU) of the 13 protein-coding genes (PCGs) were analyzed using PhyloSuite and visualized with the 'ggplot2' package (Wickham 2016) in R v. 4.1.3 (R Core Team 2023). DnaSP v. 6.0 (Rozas et al. 2017) was used to estimate the nucleotide diversity (Pi) in a sliding window analysis (a sliding window of 100 bp and a step size of 20 bp) and non-synonymous (Ka) / synonymous (Ks) substitution rates, based on the sequences of Neritidae listed in Table 1.

**Table 1.** List of 29 species and two outgroups used for phylogenetic analysis.

| Species | Family | Length (bp) | A + T (%) | Accession No. | Reference |
|---|---|---|---|---|---|
| *Nerita histrio* | Neritidae | 15538 | 65.5 | NC_068083 | Qi et al. 2023 |
| *Nerita signata* | Neritidae | 15630 | 65.3 | NC_068082 | Qi et al. 2023 |
| *Nerita plicata* | Neritidae | 15737 | 61.6 | NC_068081 | Qi et al. 2023 |
| *Nerita ocellata* | Neritidae | 15577 | 63.8 | NC_068080 | Qi et al. 2023 |
| *Nerita insculpta* | Neritidae | 15721 | 61.5 | NC_068079 | Qi et al. 2023 |
| *Nerita costata* | Neritidae | 15604 | 61.6 | NC_068078 | Qi et al. 2023 |
| *Clithon sowerbianum* | Neritidae | 15895 | 64.4 | NC_068077 | Qi et al. 2023 |
| *Clithon oualaniense* | Neritidae | 15712 | 65.7 | NC_068076 | Qi et al. 2023 |
| *Clithon retropictum* | Neritidae | 15814 | 64.9 | NC_031893 | Fukumori et al. 2016 |
| *Nerita japonica* | Neritidae | 15875 | 65.2 | MN747116 | Feng et al. 2021 |
| *Nerita albicilla* | Neritidae | 15314 | 64.5 | MK516738 | Feng et al. 2019 |
| *Nerita yoldii* | Neritidae | 15719 | 64.7 | MK395169 | Xie et al. 2019 |
| *Nerita versicolor* | Neritidae | 15866 | 61.6 | KF728890 | Arquez et al. 2014 |
| *Nerita tessellata* | Neritidae | 15741 | 64 | KF728889 | Arquez et al. 2014 |
| *Nerita fulgurans* | Neritidae | 15343 | 64.4 | KF728888 | Arquez et al. 2014 |
| *Nerita undata* | Neritidae | 15878 | 62.8 | NC_087859 | Unpublished |
| *Septaria tessellaria* | Neritidae | 15697 | 65.8 | NC_064130 | Miao et al. 2022 |
| *Clithon squarrosum* | Neritidae | 15905 | 65 | NC_062613 | Miao et al. 2022 |
| *Neritina iris* | Neritidae | 15618 | 64.3 | NC_062612 | Miao et al. 2022 |
| *Clithon corona* | Neritidae | 15975 | 64.8 | NC_062611 | Miao et al. 2022 |
| *Clithon lentiginosum* | Neritidae | 15885 | 64.8 | NC_062610 | Miao et al. 2022 |
| *Neripteron violaceum* | Neritidae | 15618 | 65.8 | NC_060871 | Unpublished |
| *Theodoxus fluviatilis* | Neritidae | 15667 | 66.1 | MT628587 | Unpublished |
| *Nerita balteata* | Neritidae | 15571 | 63.3 | MN477253 | Unpublished |
| *Nerita chamaeleon* | Neritidae | 15716 | 65.8 | MT161611 | Feng et al. 2021 |
| *Neripteron violaceum* | Neritidae | 15710 | 66.3 | KY021066 | Wang et al. 2019 |
| *Vitta usnea* | Neritidae | 15574 | 64 | KU342665 | Uribe et al. 2016 |
| *Nerita melanotragus* | Neritidae | 15261 | 63.5 | GU810158 | Castro and Colgan 2010 |
| *Neritona juttingae* | Neritidae | 15950 | 62.7 | PP958837 | This study |
| *Titiscania limacina* | Neritopsidae | 15046 | 61.8 | KU342669 | Uribe et al. 2016 |
| *Georissa bangueyensis* | Hydrocenidae | 15267 | 68.8 | KU342664 | Uribe et al. 2016 |

## Results

### Species identification

The identifying characters of the specimen fit well with *Neritona juttingae*, the description in Eichhorst (2016a), including a semi-globose shell with brown to black periostracum, with 6–9 prominent spiral cords; each spiral cord has a series of small nodules that develop into spines on the final whorl (Fig. 1).

The BLAST result of the complete mitochondrial genome showed no match with *Neritona juttingae* (isolate: ANJ3), previously submitted by Ng et al. (2016). We then extracted the full-length *COI* and 16S sequences from the mitochondrial genome for separate BLAST analyses. The *COI* sequence also did not match with *N. juttingae* (isolate: ANJ3). However, the 16S sequence showed a 99.8% identity with *Neritona juttingae* (isolate: ANJ3; 16S: KU318361.1). Upon further inspection, we found that the *COI* sequence of *N. juttingae* (isolate: ANJ3; *COI*: KU318383.1) is only 313 bp in length. Therefore, we trimmed our *COI* and 16S

sequences to a similar length as those in the database before re-running the BLAST analyses. The results then showed high identity (over 99%) for both genes with *N. juttingae* (isolate: ANJ3). All BLAST results see Suppl. material 1: table S4.

Phylogenetic trees based on both *COI* and 16S sequences consistently showed that our specimen clustered with *Neritona juttingae* (isolate: ANJ3). Therefore, based on the combined morphological, BLAST, and phylogenetic evidence, we identified our specimen as *Neritona juttingae*.

## Mitochondrial genome organization

The complete mitochondrial genome of *Neritona juttingae* is 15,950 bp in length, comprising 13 protein-coding genes (PCGs), two rRNA genes, 22 tRNA genes, and a non-coding region (NCR) measuring 818 bp. Six PCGs (*cox1*, *cox2*, *atp8*, *atp6*, *cox3*, and *nad3*) and seven tRNAs (*trnD*, *trnK*, *trnA*, *trnR*, *trnN*, *trnI*, *trnS1*, and *trnT*) are located on the heavy (H-) strand, while the remaining genes are positioned on the light (L-) strand (Table 2, Fig. 2A). Overall, the light- and heavy-strand regions within the mitochondrial genome of *N. juttingae* were concentrated and characterized by both intergenic (19 intergenic intervals, totaling 212 bp) and only one overlapping region (7 bp, between *nad4* and *nad4l*) (Table 2). This typical overlap was common in other Neritidae sequences (Feng et al. 2021; Miao et al. 2022; Qi et al. 2023). All Neritidae mitochondrial genomes exhibited a high A + T content (61.5%–66.3%) (Table 1). The A + T content of *N. juttingae* was 62.7%, with A, T, G, and C constituting 27.8%, 34.9%, 23.0%, and 14.3%, respectively. The AT-skew of the whole mitochondrial genome was negative (-0.113) and the GC-skew was positive (0.235) (Table 3).

## Nuclear rRNA gene cluster

The nuclear rRNA gene cluster (10,124 bp) contains the full complement of ribosomal RNA genes: 18S rRNA (1,818 bp), ITS1 (673 bp), 5.8S rRNA (156 bp), ITS2 (453 bp), and 28S rRNA (3,693 bp) (Fig. 2B).

## Genes and codon usage

The 13 protein-coding genes (PCGs) of *Neritona juttingae* have a total length of 11,280 bp and encode 3,760 amino acids. All 13 PCGs begin with the ATG start codon. Regarding stop codons, *nad5*, *nad4*, and *nad2* terminate with a single T, *cox1*, *cox2*, *nad6*, and *nad3* end with TAG, while the remaining genes end with TAA (Table 2). The AT- and GC-skews of the 13 PCGs are negative, at -0.177 and -0.032, respectively (Table 3). Six PCGs (*cytb*, *nad1*, *nad4*, *nad4l*, *nad5*, and *nad6*) exhibit negative GC-skew values, whereas the remaining seven PCGs show positive values. The 12S rRNA gene (866 bp) is located between the *trnV* and *trnM* genes, while the 16S rRNA gene (1,296 bp) is positioned between *trnL1* and *trnV* (Table 2, Fig. 2A). A total of 22 tRNA genes, ranging in length from 64 to 72 bp, were identified in the mitochondrial genome of *N. juttingae*.

The relative synonymous codon usage (RSCU) values for the PCGs in the mitochondrial genome were also analyzed (Fig. 3, Suppl. material 1: table S5). The most frequently used amino acids are *Leu* (16.0%), *Ser* (9.25%), *Phe* (7.79%), and *Val* (7.68%). The least common amino acids are *Cys* (1.47%), *Arg*

**Table 2.** Features of the *N. juttingae* mitochondrial genome.

| Gene | Position | | Length (bp) | Amino | Start/stop codon | Anticodon | Intergenic region | Strand |
|---|---|---|---|---|---|---|---|---|
| | From | To | | | | | | |
| *cox1* | 1 | 1548 | 1548 | 516 | ATG/TAG | | 11 | H |
| *cox2* | 1560 | 2249 | 690 | 230 | ATG/TAG | | 2 | H |
| *trnD* | 2252 | 2317 | 66 | | | GTC | 0 | H |
| *atp8* | 2318 | 2482 | 165 | 55 | ATG/TAA | | 6 | H |
| *atp6* | 2489 | 3190 | 702 | 234 | ATG/TAA | | 22 | H |
| *trnF* | 3213 | 3280 | 68 | | | GAA | 0 | L |
| *nad5* | 3281 | 4994 | 1714 | 572 | ATG/T | | 0 | L |
| *trnH* | 4995 | 5060 | 66 | | | GTG | 9 | L |
| *nad4* | 5070 | 6426 | 1357 | 453 | ATG/T | | -7 | L |
| *nad4l* | 6420 | 6713 | 294 | 98 | ATG/TAA | | 4 | L |
| *trnT* | 6718 | 6785 | 68 | | | TGT | 18 | H |
| *trnS2* | 6804 | 6868 | 65 | | | TGA | 5 | L |
| *cytb* | 6874 | 8010 | 1137 | 379 | ATG/TAA | | 2 | L |
| *nad6* | 8013 | 8519 | 507 | 169 | ATG/TAG | | 1 | L |
| *trnP* | 8521 | 8586 | 66 | | | TGG | 0 | L |
| *nad1* | 8587 | 9519 | 933 | 311 | ATG/TAA | | 0 | L |
| *trnL2* | 9520 | 9587 | 68 | | | TAA | 0 | L |
| *trnL1* | 9588 | 9657 | 70 | | | TAG | 0 | L |
| 16S | 9658 | 10953 | 1296 | | | | 0 | L |
| *trnV* | 10954 | 11021 | 68 | | | TAC | 0 | L |
| 12S | 11022 | 11887 | 866 | | | | 0 | L |
| *trnM* | 11888 | 11954 | 67 | | | CAT | 14 | L |
| *trnY* | 11969 | 12036 | 68 | | | GTA | 4 | L |
| *trnC* | 12041 | 12104 | 64 | | | GCA | 0 | L |
| *trnW* | 12105 | 12170 | 66 | | | TCA | 0 | L |
| *trnQ* | 12171 | 12239 | 69 | | | TTG | 0 | L |
| *trnG* | 12240 | 12304 | 65 | | | TCC | 12 | L |
| *trnE* | 12317 | 12382 | 66 | | | TTC | 818 | L |
| *cox3* | 13201 | 13980 | 780 | 260 | ATG/TAA | | 29 | H |
| *trnK* | 14010 | 14076 | 67 | | | TTT | 33 | H |
| *trnA* | 14110 | 14177 | 68 | | | TGC | 29 | H |
| *trnR* | 14207 | 14275 | 69 | | | TCG | 5 | H |
| *trnN* | 14281 | 14352 | 72 | | | GTT | 2 | H |
| *trnI* | 14355 | 14422 | 68 | | | GAT | 0 | H |
| *nad3* | 14423 | 14776 | 354 | 118 | ATG/TAG | | 4 | H |
| *trnS1* | 14781 | 14848 | 68 | | | GCT | 0 | H |
| *nad2* | 14849 | 15950 | 1102 | 368 | ATG/T | | 0 | H |

(1.71%), *Gln* (1.92%), and *Asp* (2.08%) (Fig. 3A, Suppl. material 1: table S5). RSCU analysis indicates that the most frequently used codons are CGA (*Arg*), UCU (*Ser*), and CCU (*Pro*), while CUG (*Leu*), AGG (*Ser*), and ACG (*Thr*) have the lowest frequencies (Fig. 3B, Suppl. material 1: table S5).

## Phylogenetic analysis

The gene arrangement of the 13 PCGs and 2 rRNAs within the family Neritidae is completely consistent (Fig. 4A, Suppl. material 3) and cannot provide useful phylogenetic information. A total of ten ML and BI trees were

**Table 3.** Composition and skewness of the *N. juttingae* mitochondrial genome.

| Regions | A (%) | T (%) | C (%) | G (%) | A + T (%) | AT-skew | GC-skew | Size (bp) |
|---|---|---|---|---|---|---|---|---|
| Full genome | 27.8 | 34.9 | 14.3 | 23.0 | 62.7 | -0.113 | 0.235 | 15950 |
| PCGs | 25.2 | 36.0 | 20.0 | 18.8 | 61.2 | -0.177 | -0.032 | 11280 |
| tRNAs | 31.1 | 32.3 | 15.3 | 21.3 | 63.4 | -0.018 | 0.164 | 1482 |
| rRNAs | 36.2 | 29.4 | 17.3 | 17.1 | 65.6 | 0.103 | -0.005 | 2162 |
| 1st codon | 25.9 | 29.7 | 18.1 | 26.4 | 55.6 | -0.068 | 0.185 | 3760 |
| 2nd codon | 18.1 | 42.8 | 22.7 | 16.4 | 60.9 | -0.407 | -0.162 | 3760 |
| 3rd codon | 31.6 | 35.5 | 19.2 | 13.6 | 67.1 | -0.058 | -0.172 | 3760 |
| 12S | 35.2 | 28.9 | 17.0 | 18.9 | 64.1 | 0.099 | 0.055 | 866 |
| 16S | 36.8 | 29.8 | 17.5 | 15.9 | 66.6 | 0.105 | -0.048 | 1296 |
| *atp6* | 20.1 | 42.2 | 15.8 | 21.9 | 62.3 | -0.355 | 0.162 | 702 |
| *atp8* | 25.5 | 41.2 | 12.1 | 21.2 | 66.7 | -0.236 | 0.273 | 165 |
| *cox1* | 20.6 | 40.2 | 15.9 | 23.3 | 60.8 | -0.323 | 0.188 | 1548 |
| *cox2* | 23.3 | 37.2 | 14.6 | 24.8 | 60.5 | -0.230 | 0.257 | 690 |
| *cox3* | 21.0 | 38.7 | 14.9 | 25.4 | 59.7 | -0.296 | 0.261 | 780 |
| *cytb* | 28.1 | 31.2 | 25.8 | 15.0 | 59.3 | -0.053 | -0.266 | 1137 |
| *nad1* | 28.8 | 33.0 | 23.4 | 14.8 | 61.8 | -0.068 | -0.225 | 933 |
| *nad2* | 19.2 | 42.6 | 10.7 | 27.4 | 61.8 | -0.378 | 0.438 | 1102 |
| *nad3* | 15.5 | 46.6 | 9.3 | 28.5 | 62.1 | -0.500 | 0.507 | 354 |
| *nad4* | 29.8 | 32.3 | 26.4 | 11.6 | 62.1 | -0.040 | -0.390 | 1357 |
| *nad4l* | 33.0 | 28.9 | 22.1 | 16.0 | 61.9 | 0.066 | -0.161 | 294 |
| *nad5* | 29.7 | 30.2 | 27.4 | 12.7 | 59.9 | -0.009 | -0.368 | 1714 |
| *nad6* | 29.6 | 35.3 | 21.7 | 13.4 | 64.9 | -0.088 | -0.236 | 507 |

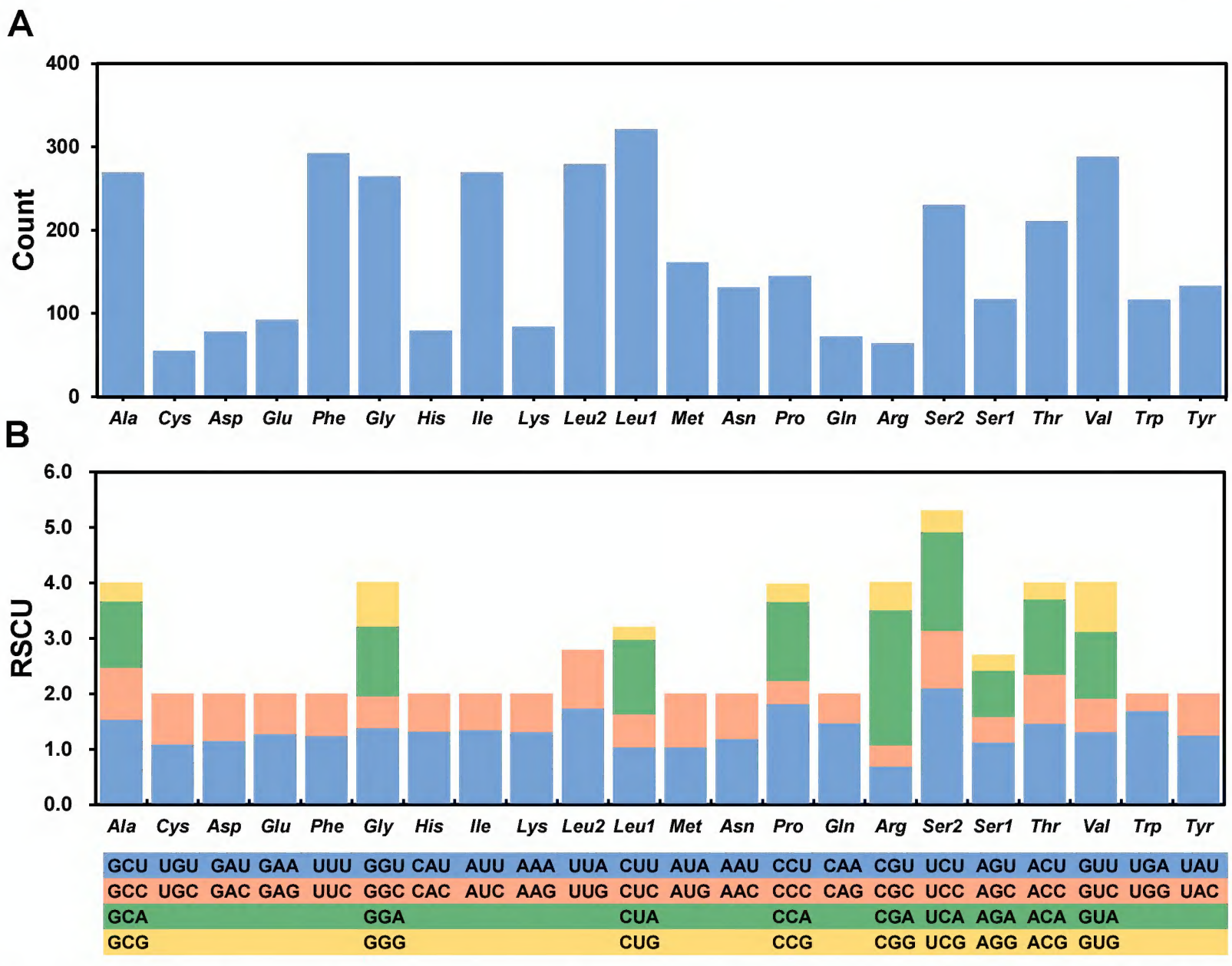
A
Count
400
300
200
100
0
Ala Cys Asp Glu Phe Gly His Ile Lys Leu2 Leu1 Met Asn Pro Gln Arg Ser2 Ser1 Thr Val Trp Tyr
B
RSCU
6.0
5.0
4.0
3.0
2.0
1.0
0.0
Ala Cys Asp Glu Phe Gly His Ile Lys Leu2 Leu1 Met Asn Pro Gln Arg Ser2 Ser1 Thr Val Trp Tyr
GCU UGU GAU GAA UUU GGU CAU AUU AAA UUA CUU AUA AAU CCU CAA CGU UCU AGU ACU GUU UGA UAU
GCC UGC GAC GAG UUC GGC CAC AUC AAG UUG CUC AUG AAC CCC CAG CGC UCC AGC ACC GUC UGG UAC
GCA GGA CUA CCA CGA UCA AGA ACA GUA
GCG GGG CUG CCG CGG UCG AGG ACG GUG

**Figure 3.** Amino acid composition (**A**) and relative synonymous codon usage (**B**) of the *N. juttingae* mitochondrial genome. The codon families are provided under the x-axis.

inferred from five mitochondrial genome datasets of Neritidae. These ten trees were grouped into four clusters, representing four distinct tree types (see Fig. 4B–F, Suppl. material 4). The most common topology, Cluster 1, is shown in Fig. 4A, C. The differences between Cluster 1 and Clusters 2–3 mainly lie in the sequences of *Vitta usnea* (KU342665), *Nerita melanotragus* (GU810158), and the outgroup species *Titiscania limacina* (KU342669). The relationships within genera are generally consistent except for *Nerita melanotragus* (GU810158), which forms a distinct clade in Cluster 2. Three genera, *Neritina*, *Septaria*, and *Neripteron*, clustered together with each other in all four clusters. The positions of *Theodoxus fluviatilis* (MT628587) and *Neritona juttingae* (PP958837) form two distinct lineages from other genera, although there are some differences between the four clusters (Fig. 4).

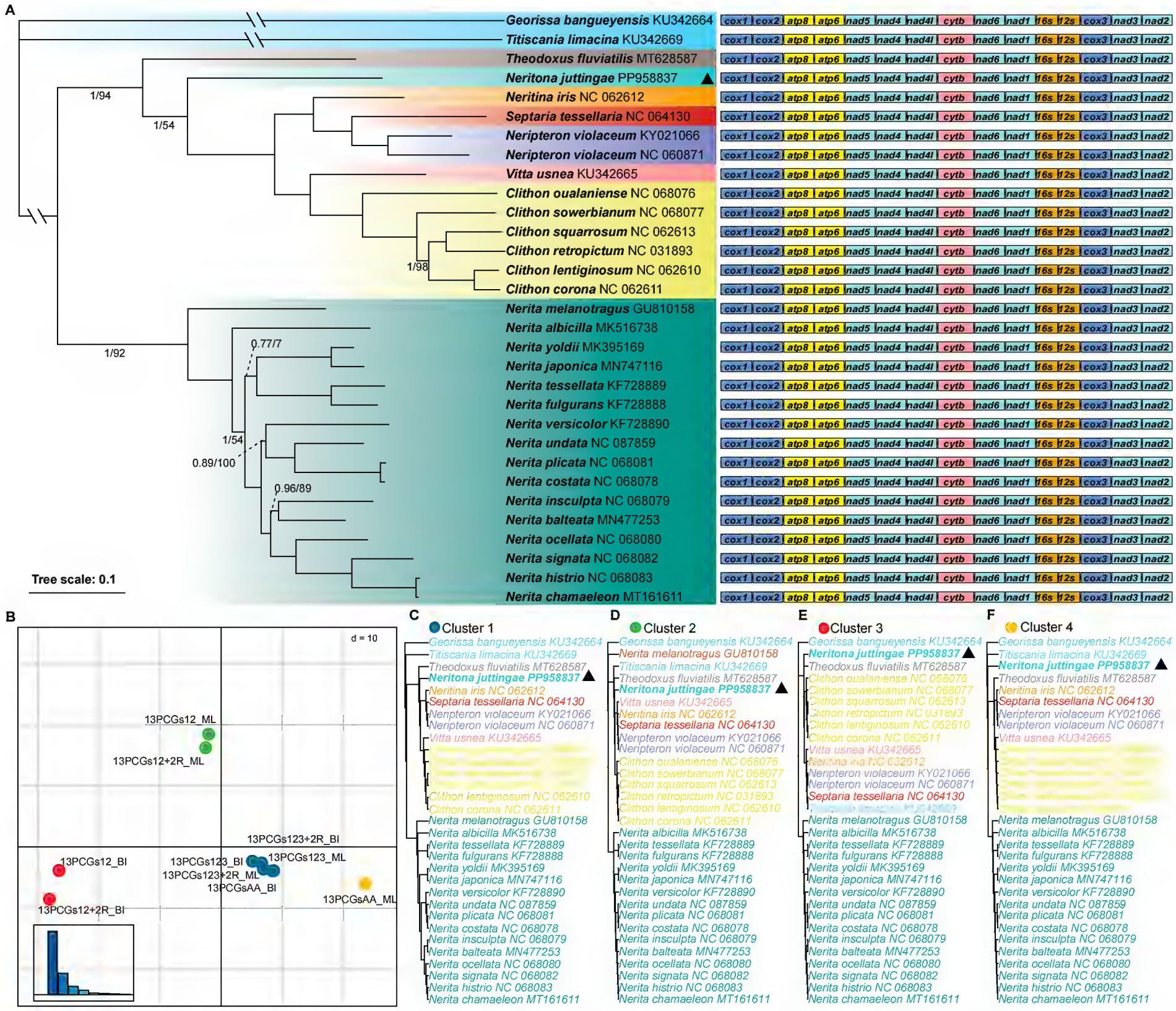


**Figure 4.** An analysis of phylogenies from five mitochondrial genome datasets. **A.** Maximum likelihood (ML) and Bayesian inference (BI) tree of Neritidae based on the dataset 13PCGs123. The GenBank accession numbers used are listed after the species names. The scale bar (0.1) corresponds to the estimated number of substitutions per site. Numbers at nodes are statistical support values for "BI posterior probabilities/ML bootstrap support". "Unlabeled" denotes 100% bootstrap support. Color-coded clades are different genera within Neritidae. The gene order is shown to the right (13 PCGs and two rRNAs); **B.** Two-dimensional MDS plot of ten trees, colored by different clusters; **C–F.** For each cluster identified in (**B**), a representative tree was selected.

Subsequent phylogenetic interpretations were based on the dataset 13PCGs123. Phylogenetic analyses using the 13PCGs123 dataset from the available mitochondrial genomes (29 taxa in Neritidae, including 8 genera) support that *Neritona juttingae* belongs to a separate genus.

The phylogenetic tree comprises 29 sequences from 28 species of Neritidae, belonging to eight genera (Fig. 4A). The tree can be divided into two primary clades: Clade A comprises seven genera (*Theodoxus*, *Neritona*, *Neritina*, *Septaria*, *Neripteron*, *Vitta*, and *Clithon*), and Clade B comprises only one genus, *Nerita*. The genus *Neritona* is located inside Clade A, forming a distinct clade, which is most closely related to a clade comprising five freshwater or brackish genera (*Neritina*, *Septaria*, *Neripteron*, *Vitta*, and *Clithon*) with strong support (BS = 100%, PP = 1).

The *COI*+16S tree includes 114 species from 12 genera of Neritidae, and two outgroups (Fig. 5, Suppl. material 5). The structure of the *COI*+16S tree is similar to the mitochondrial genome tree, and both can be divided into two clades, comprising about the same genera. Two sequences of *Neritona juttingae* are also located inside Clade A, forming a distinct clade, which is most closely related to the other 10 genera of Clade A.

The *COI*_ex tree includes 1480 sequences of Neritidae. This tree is not clustered into two distinct branches like the previous two, but the genus *Nerita* still forms a generally distinct clade. Two sequences of *N. juttingae* form a distinct clade, too.

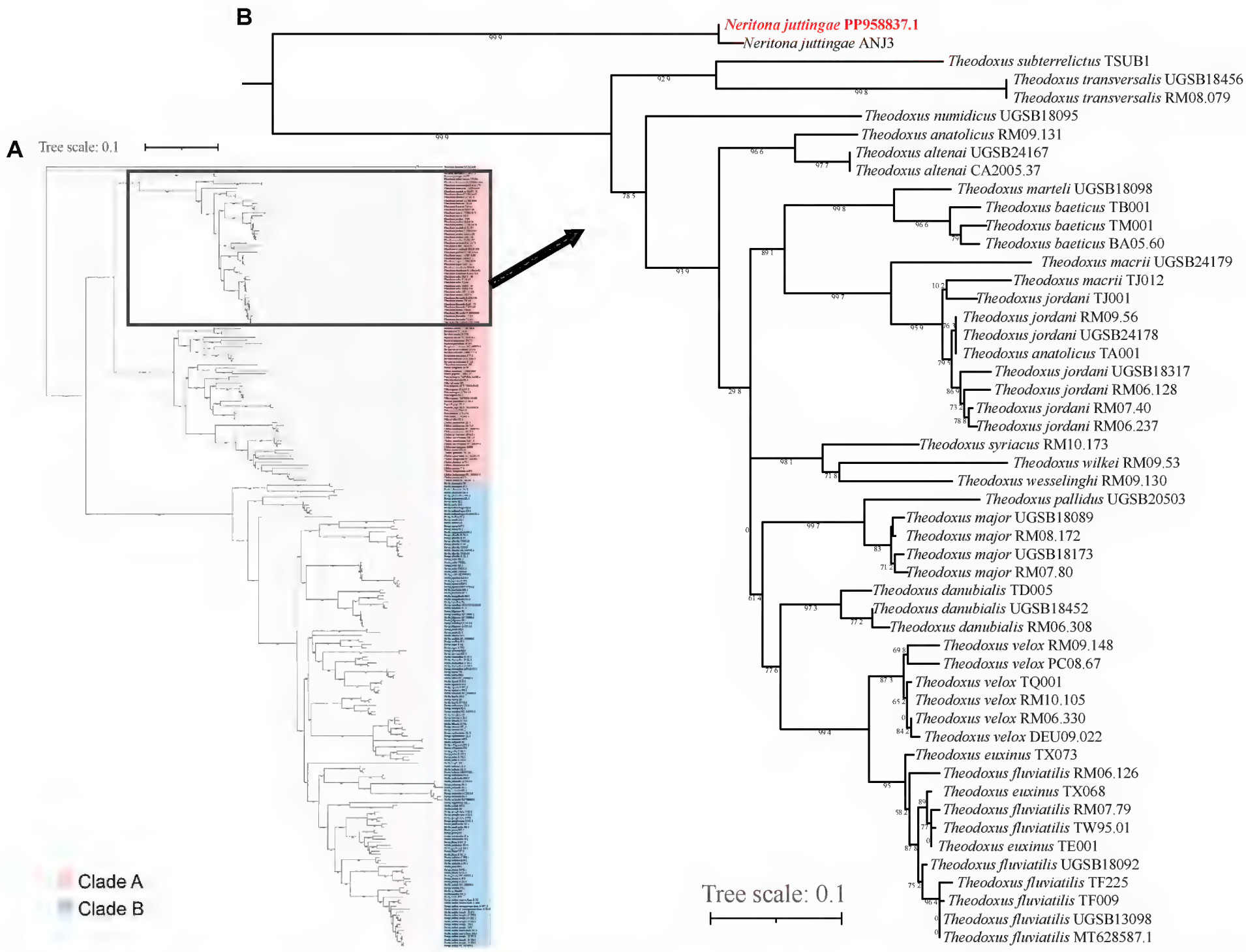


**Figure 5. A.** ML tree based on the *COI*+16S dataset (Suppl. material 5); **B.** Enlarged version of the branch containing *Neritona juttingae* sequences. The *N. juttingae* sequence reported in this study are highlighted in red text.

Sequences of the genus *Neritona* do not cluster together. Two sequences of *N. juttingae* form a distinct group close to the genus *Theodoxus*, one sequence of *N. granosa* (AF364065) is close to *Neripteron vespertinum* AF364066, and other *N. granosa* sequences form a monophyletic group (Suppl. material 6).

## Nucleotide diversity and evolutionary rate analysis

The nucleotide diversity (Pi) analysis was conducted using concatenated alignments of 13 PCGs and two rRNAs of 29 Neritidae species. The sequence variation ratio exhibits variable nucleotide diversity between the Neritidae, with Pi values for the 100 bp windows ranging from 0.024 to 0.346. 12S (Pi = 0.114), *cox1* (0.142), 16S (0.145), and *cox2* (0.148) exhibit a comparatively low sequence variability, whereas *nad6* (0.231), *nad2* (0.214), *nad4* (0.208), and *nad5* (0.208) have a comparatively high sequence variability (Fig. 6A). This is broadly corroborated by the non-synonymous/synonymous (Ka/Ks) ratio analysis, which shows that *cox1* (Ka/Ks = 0.025), *cytb* (0.030), *nad1* (0.032), and *atp6* (0.032) are evolving comparatively slowly, whereas *atp8* (0.116), *nad2* (0.070), and *nad6* (0.066) are evolving comparatively fast (Fig. 6B).

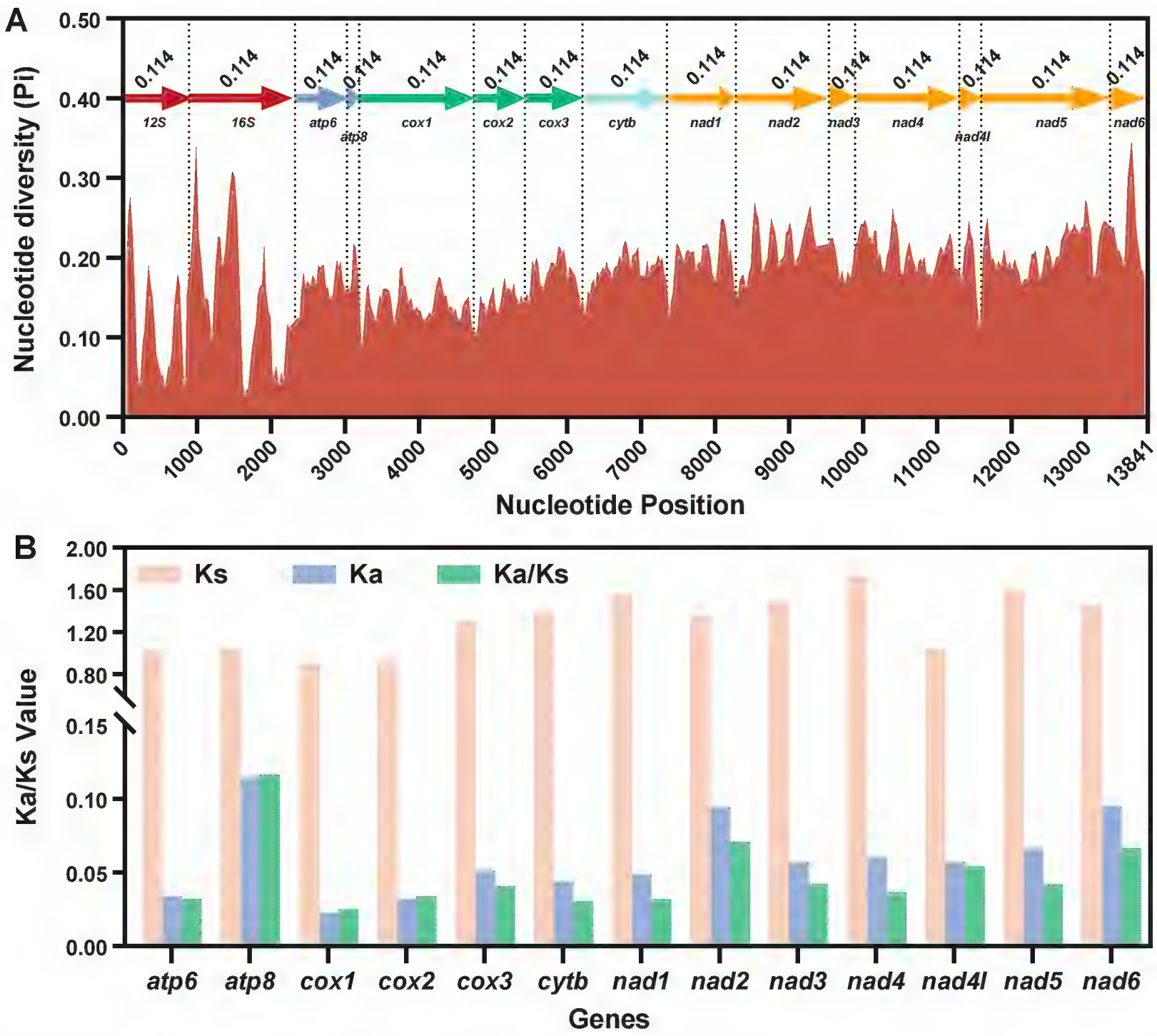


**Figure 6.** Nucleotide diversity analysis (**A**) of two rRNAs + 13 PCGs and Ka/Ks rates (**B**) of 13 PCGs based on 29 Neritidae species. The Pi values for the 13 PCGs + two rRNAs are shown on the graph. The red line represents the value of nucleotide diversity (Pi) (window size = 100 bp, step size = 20 bp). The pink, purple, and green columns represent the values of Ks, Ka, and Ka/Ks, respectively.

## Discussion

In this study, our results well supported the classification of this family into two subfamilies (Neritininae and Neritinae), which is consistent with previous mitochondrial genome-based phylogenetic analysis studies (Feng et al. 2021; Miao et al. 2022; Qi et al. 2023). These studies consistently reveal two distinct clades: one clade (Fig. 4, Clade A) includes genera of the subfamily Neritininae, which primarily inhabit freshwater or brackish environments and typically exhibit amphidromous life histories; the other clade (Fig. 4, Clade B) consists exclusively of the marine genus *Nerita*, representing the subfamily Neritinae (Ford 1979; Eichhorst 2016a, b).

However, within Neritininae, the phylogenetic relationships remain unstable, as inconsistencies are observed across our five mitochondrial genome datasets (Fig. 4). These discrepancies may be attributable to heterogeneous evolutionary rates among mitochondrial genes (Mueller 2006), long-branch attraction (Masta et al. 2010), and incomplete lineage sorting (Ballesteros and Sharma 2019). Increasing the number of species in the sample is expected to alleviate the long branch attraction phenomenon (Ontano et al. 2021) and help detect incomplete phylogenetic sorting (Maddison and Knowles 2006). Four clusters of tree structure were concluded from the ten trees based on five datasets. The relationship between genera varies a lot, although the genus-level clades are generally stable among the four clusters. The relatively stable relationship between *Neritina*, *Septaria*, and *Neripteron* suggested the three genera might have more closer relationship with each other than with other genera. Notably, the distinct position of *Nerita melanotragus* (GU810158) in Cluster 2 is similar to Feng et al. (2021). The mitochondrial gene order is highly conserved within Neritidae, which cannot provide phylogenetic information. These results may suggest the limitation of mitochondrial genome in explaining the relationship within Neritidae. Moreover, the current phylogenetic trees are still insufficient to explain the relationship between habitat and evolution in Neritidae. Holthuis (1995) proposed that there are at least 12 shifts between marine, brackish, and freshwater habitats in Cycloneritida. Qi et al. (2023) also mentioned that Neritidae moved repeatedly between different habitats. To solve this problem, importing more taxa and integrating nuclear genomic data is still required to explain the phylogenetics of all sixteen genera of Neritidae.

The monophyly of the genus *Neritona* could not be robustly assessed in this study due to the inclusion of only a single species (*N. juttingae*) within the molecular dataset. Phylogenetic analyses consistently placed *Neritona juttingae* as a distinct lineage within Clade A in both mitochondrial genome and *COI*+16S trees (Figs 4, 5), suggesting *N. juttingae* belongs to a separate genus of Neritininae. Considering that *N. juttingae* is the only species of this genus in the tree, as well as the unusually wide distribution of species in the genus (from Southeast Asia to Hawaii) and the large morphological differences within the genus, monophyly of the genus *Neritona* could not be evaluated due to the inclusion of only one of its six valid species (*N. juttingae*) in our analysis. The distinct phylogenetic position of *N. juttingae* suggests that the genus, as currently defined, may not be monophyletic, but this hypothesis requires testing with data from the type species (*N. labiosa*) and other congeners. Eichhorst (2016a) even hypothesized that *N. juttingae* might belong to a new genus distinct from

*Neritona* due to its unique appearance, because it is the only nerite species with multiple rows of spines. This assumption is possible because the sequences of *N. juttingae* and *N. granosa* do not cluster together in the *COI*_ex tree. However, although the *COI*_ex dataset contains many sequences, it provides limited phylogenetic resolution due to their short lengths. In summary, additional sampling and more complete mitochondrial genomes are needed in future studies to clarify the phylogenetic relationships of *N. juttingae*, and potentially, the entire genus *Neritona*.

We noticed that the two sequences of *Neripteron violaceum* (Gmelin, 1791) (KY021066 and NC_060871) exhibit an unusual level of consistency (89.94% identity), and the two sequences in the *COI*+16S tree are located in different subclades. KY021066 is clustered with the other two *N. violaceum* sequences, and NC_060871 is clustered with an *N. auriculatum* sequence. Therefore, we suppose the two sequences may not correspond to the same species. This phenomenon suggests there is a misidentification or cryptic species. Since the references for both sequences do not provide specimen images and morphological descriptions, this problem cannot be resolved at present. Given that some sequences in the database have been proven to be misidentified (Xu et al. 2024; Xu et al. 2025), we recommend that when uploading sequences to the database, the references should be accompanied by clear images, morphological descriptions, and acquisition information of the specimens. Besides, three of the sequences were incorrectly labeled as their invalid synonyms in the GenBank: *Neripteron violaceum* (KY021066) is labeled as *Neritina violacea*, belonging to another genus (Eichhorst 2016b), and *Septaria tessellaria* (NC064130) and *Nerita signata* (NC068082) are labeled as *S. lineata* and *N. reticulata*, respectively (MolluscaBase 2025). Similar situations are also observed in the *COI*_ex dataset. Incorrectly labeled and misidentified sequences in databases may lead to erroneous phylogenetic results. Here, we recommend that the sequences in the public databases be checked carefully before phylogenetic analysis, especially their identification and taxonomic status. Our study provided definitive molecular references for *N. juttingae*, which is particularly relevant for the ornamental pet trade, where accurate species identification is fundamental to sustainable sourcing, consumer transparency, and effective conservation management of popular species.

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## Additional information

### Conflict of interest

The authors have declared that no competing interests exist.

### Ethical statement

No ethical statement was reported.

### Use of AI

No use of AI was reported.

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## Author contributions

Conceptualization and project administration: D. Y. Y. and X. C. L.; Data curation: Y. Z. M., S. Zeng and D. Y. Y.; Formal analysis: S. Zeng, D. Y. Y., Y. Z. M., S. Zhong, and Y. B. X; Funding acquisition: X. C. L.; Investigation: Y. Z. M. and D. Y. Y.; Methodology: D. Y. Y. and Y. Z. M.; Supervision: D. Y. Y.; Visualization: S. Zeng and Y. Z. M.; Writing (original draft): X. C. L., Y. Z. M., S. Zeng, and D. Y. Y.; Writing (review & editing): All authors.

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## Data availability

All of the data that support the findings of this study are available in the main text or Supplementary Information.

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## Supplementary material 1

### Supplementary tables

Authors: Xiuchun Lin, Yuanzheng Meng, Sheng Zeng, Shen Zhong, Yibin Xu, Deyuan Yang

Data type: xlsx

Explanation note: **table S1.** List of 257 sequences used for *COI+*16S dataset; **table S2.** Original and TrimAl lengths of 13 PCGs, 2 rRNAs, and 13 PCGsAA sequences; **table S3.** Best partitioning schemes and models based on different datasets for maximum likelihood and Bayesian inference analysis; **table S4.** BLAST results of different genes or region (only show top 10 results); **table S5.** Codon numbers and relative synonymous codon usage (RSCU) of 13 PCGs in the *Neritona juttingae* mitochondrial genome.

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Link: https://doi.org/10.3897/zookeys.1269.164112.suppl1

## Supplementary material 2

### ML and BI from all NCBI mitochondrial genomes (2025-01-10) of Neritidae

Authors: Xiuchun Lin, Yuanzheng Meng, Sheng Zeng, Shen Zhong, Yibin Xu, Deyuan Yang

Data type: pdf

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## Supplementary material 3

### All the mt genes order of Neritidae

Authors: Xiuchun Lin, Yuanzheng Meng, Sheng Zeng, Shen Zhong, Yibin Xu, Deyuan Yang

Data type: pdf

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## Supplementary material 4

### 10 trees from different datasets and tree-building methods

Authors: Xiuchun Lin, Yuanzheng Meng, Sheng Zeng, Shen Zhong, Yibin Xu, Deyuan Yang

Data type: zip

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Link: https://doi.org/10.3897/zookeys.1269.164112.suppl4

## Supplementary material 5

### ML tree based on the *COI*+16S dataset

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Data type: pdf

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## Supplementary material 6

### ML tree based on the *COI_ex* dataset

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Data type: pdf

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